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LOCALIZATION AND CHARACTERIZATION OF LUMINESCENT CELLS IN *OPHIOPSILA CALIFORNICA* AND *AMPHIPHOLIS SQUAMATA* (ECHINODERMATA: OPHIUROIDEA)

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The identification of light-emitting cells is central to the understanding of the control of bioluminescence. In many phyla little is known of the morphology and organization of the luminescent cells, primarily because techniques have not been available for spatially detecting low light levels. Within the echinoderms, luminescent representatives of the class Ophiuroidea have long been recognized (Harvey, 1952). However, luminescence has recently been observed in certain Holothuroidea, Crinoidea, and Asteroidea, as well as confirmed in the Ophiuroidea (Herring, 1974).

In the first decade of this century, a concentrated but inconclusive effort was made to locate the photogenic cells in ophiuroids (Sterzinger, 1907; Reichensperger, 1908a; Trojan, 1909a; Sokolow, 1909). The results of most of these investigations led to the opinion that some type of epidermal gland cell was involved, but different cells were implicated by various authors. Very little information appeared subsequently until Buchanan (1963) reported further details of the luminescent cells first described by Reichensperger (1908a) in *Amphipholis* (= *Amphiura*) *squamata* and *Amphiura filiformis*. He described this cell type as being comprised of a pyriform, nucleated cell body deep within the spine. The cell body tapered to an elongate duct which could reach long distances to the surface cuticle. The resemblance of these large cells, termed photocytes, to neighboring mucous glands suggested that the cells were of glandular origin (Buchanan, 1963). Extracellular luminescence was suggested by early observers (Reichensperger, 1908b; Trojan, 1909a), who indicated that the cells communicated with the exterior through the duct. However, there was no physiological evidence to support the theory of extracellular luminescence. Reichensperger (1908b) and Sokolow (1909) recognized that this cell did not perfectly match the distribution of observed luminescent sites. Herring (1974) also reported an inability to find any epidermal gland cell which corresponded to the areas he found to be luminescent in ophiuroids.

The purpose of the present paper is to describe: the general distribution of luminescent areas; the specific cellular sources of luminescence; and the luminescence emission spectra of *Ophiopsila californica* Clark, 1921 and *Amphipholis squamata* (Delle Chiaje, 1828). A preliminary report of this work has been published (Brehm, Morin, and Reynolds, 1973).

Materials and Methods

Two species of ophiuroids were used in this study, *Ophiopsila californica* and *Amphipholis* (= *Axiognathus*, see Thomas, 1966 and Clark, 1970) *squamata*.

Both species were collected near the Santa Catalina Marine Biological Laboratory, California, using SCUBA at night from depths of 10 to 30 meters. *Amphipholis squamata* was also collected in shallow water at Palos Verdes Point, California, and at Odiorne Point, New Hampshire. *Ophiopsila californica* is large, and each arm can reach a length of 20 centimeters from disc to arm tip. *Amphipholis squamata* is small, and the arms only reach a length of approximately 2 centimeters. The animals were maintained at U.C.L.A. in a closed circulating seawater system at 12° C.

The distribution of luminescence in *O. californica* was studied with an image intensifier coupled to a video tape deck and viewed on a television monitor. The detailed methodology of this technique has been described (Reynolds, 1972). A second method for identifying luminescent areas involved isolating and testing specific arm parts for luminescence. The parts were chemically excited with 0.54 M KCl (isotonic to sea water) and light production was monitored using standard photometric techniques.

The luminescent sites become fluorescent after stimulation. This fluorescence was used to study the distribution of luminescence. The advantage of observing fluorescence is two-fold. First, the luminescence lasts only milliseconds and is therefore difficult to observe. The fluorescence, on the other hand, can be observed easily, because it lasts for as long as the stimulating light is maintained. Secondly, fluorescence is retained even after the tissue has been appropriately fixed and sectioned. Therefore, it is possible to see cellular detail. To prepare histological sections for fluorescence microscopy, the tissue was excited to luminesce in 0.54 M KCl. It was important that the tissue was not anesthetized (in 0.36 M $MgCl_2$ isotonic to sea water or Ca-free sea water) before the KCl treatment, since anesthetized tissue did not fluoresce.

After 12 hours in 5% formalin the arms were placed in 50 mM EDTA for approximately 72 hours until decalcification of the spines and plates was complete. The arms were dehydrated in ethanol, cleared in toluene, embedded in Tissuemat (56–58° C), and cross sections were cut between 8–20 μm thickness. These sections were stained with Harris hematoxylin and eosin, toluidine blue, thionin, or Holme's silver stain and mounted in Permount. Through each stage of this procedure, most of the natural fluorescence of the photogenic material was preserved. Fluorescence was observed through a 530 nm barrier filter following excitation from a xenon source with a Leitz BG-12 (blue) filter.

The luminescent emission spectra of *O. californica* and *A. squamata* were measured with an image intensifier coupled to a spectrophotometer and analyzed both by computer (Gruner, 1973) and a Joyce Loebl microdensitometer.

Results

Distribution of the photocytes

Ophiopsila californica. A previous abstract on *Ophiopsila californica* (Brehm *et al.*, 1973) reported that image intensification showed the localization of luminescent sites. The following parts of each arm segment were demonstrated to be luminescent: spines, lateral plates, ventral plate and tentacular scales (Fig. 1). The aboral radial shields and oral plates were reported to be the only luminescent

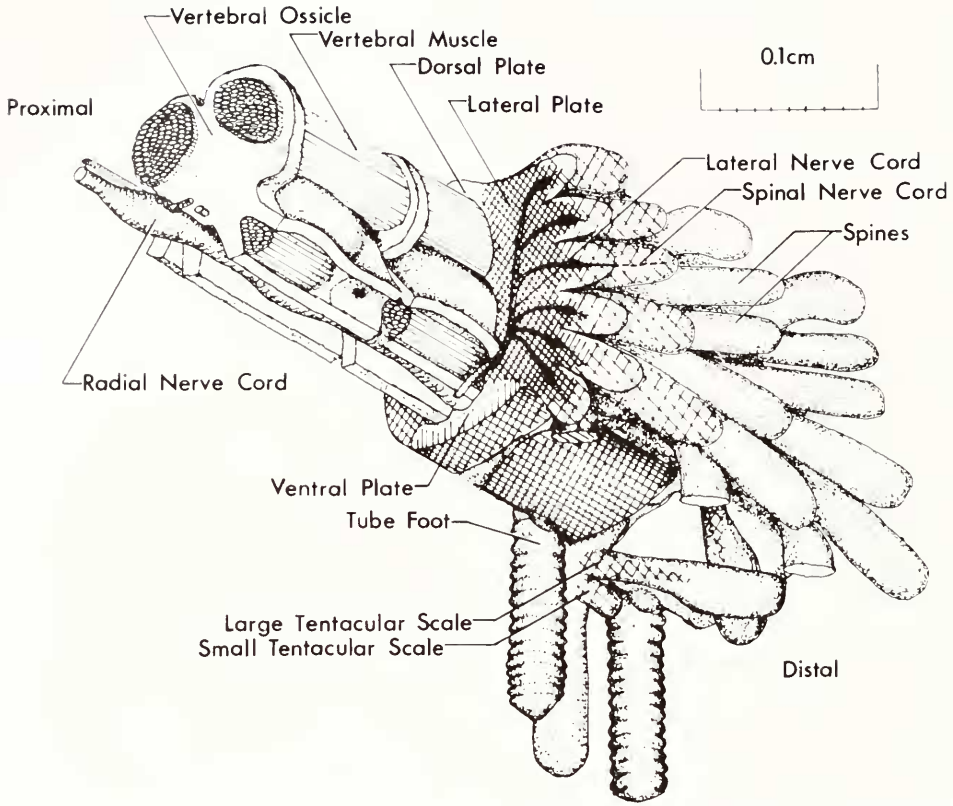

FIGURE 1. Reconstruction of five segments of an *Ophiopsila californica* arm. Luminescent areas are represented by the cross-hatching, with closer cross-hatching indicating a greater concentration of photocytes. See text for description.

components of the disc. Subsequently, these observations were verified by photometrically testing isolated parts of each segment upon addition of 0.54 M KCl. Luminescence in the whole animal was elicited by either electrical, chemical, or mechanical stimulation, and in all cases the sites of light production were identical. There was no indication of extracellular luminescence, even with chemical stimulation by application of KCl or distilled water. All of the light appeared to be emitted intracellularly from photocytes within the trabecular skeleton.

Fluorescence microscopy was also used to identify the distribution of the luminescence. Fluorescence could be observed in both *O. californica* and *A. squamata* only after the animals were stimulated to luminesce (Figs. 2 and 4). Generally, mechanical or electrical stimulation led to only very limited fluorescence, even when accompanied by intense luminescence. KCl, isotonic to sea water, was found to produce intense luminescence as well as fluorescence. Simultaneous microscopic observation and delivery of KCl allowed a direct comparison of luminescent and fluorescent areas. The fluorescent distribution corresponds precisely to the

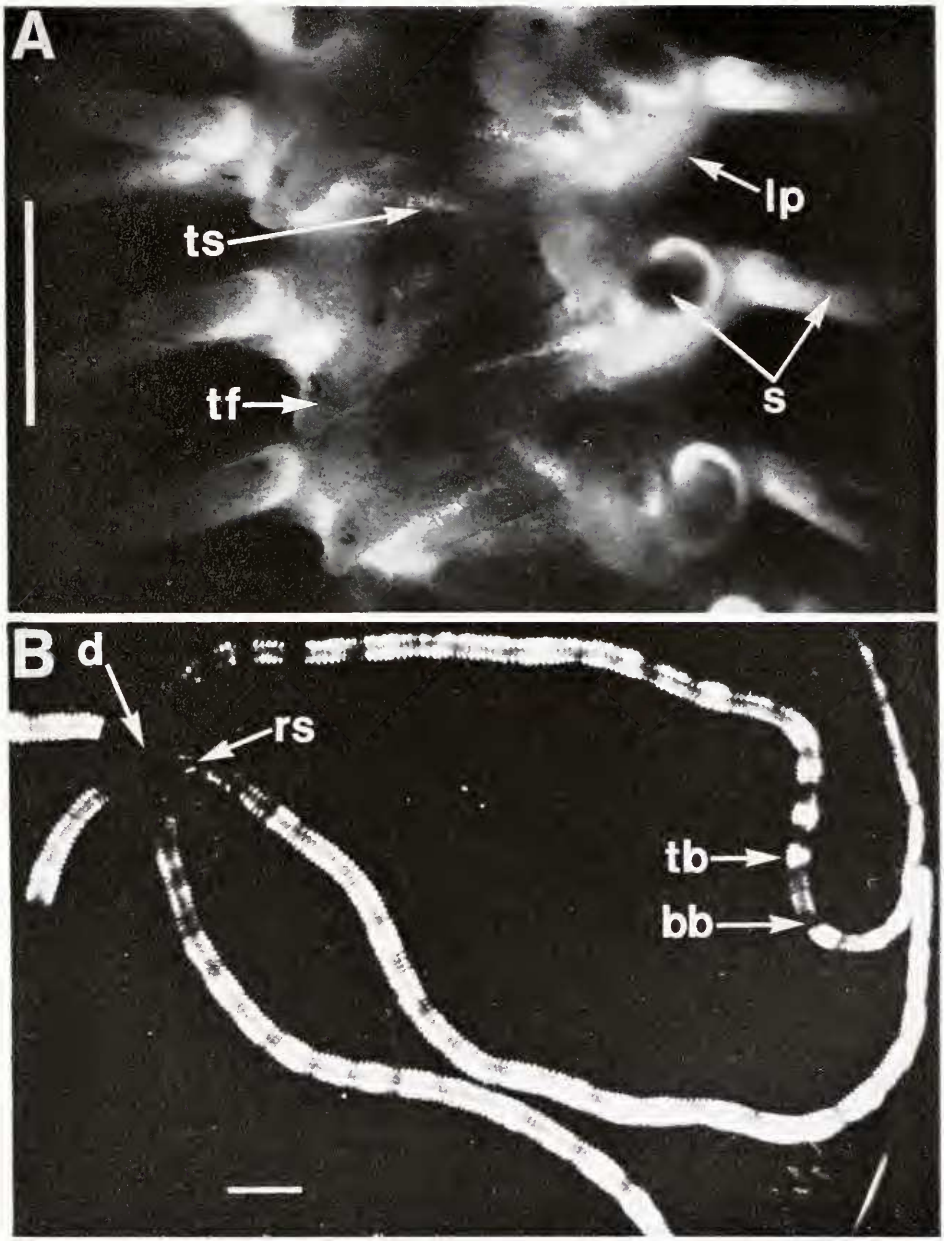

FIGURE 2. A. A ventral view of the fluorescence within three segments of an *Ophiopsila californica* arm. The arm was pretreated with 0.54 M KCl. The tube feet (tf), spines (s), tentacular scales (ts), and proximal end of the lateral plate (lp) are shown. The bar represents 1 mm. B. A luminescing *Ophiopsila californica* which illustrates the banding pattern of luminescence along the arm. Brown bands (bb) and tan bands (tb) can be discerned. Except for one pair of radial shields (rs), the disc is dark. The bar represents 1 cm. The photograph was taken with Tri-X film with an exposure time of one second.

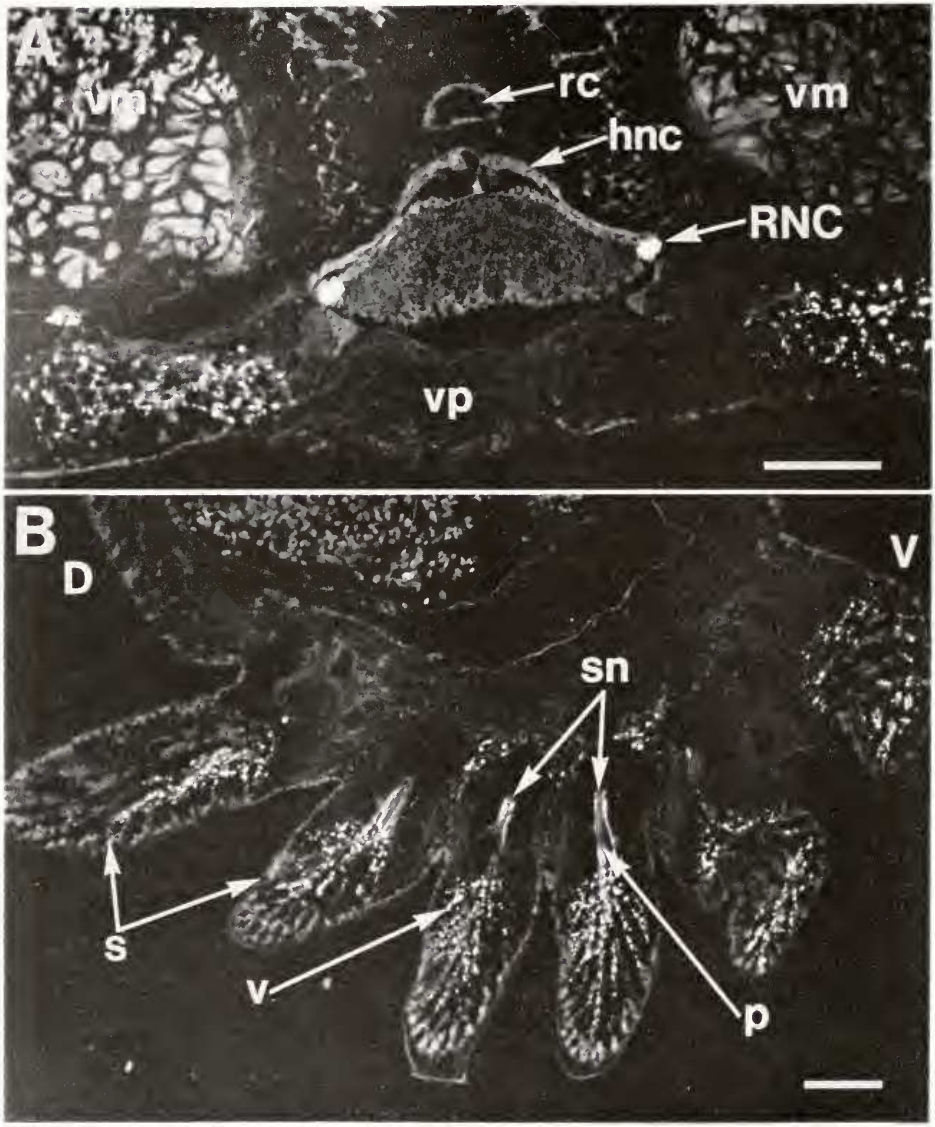

FIGURE 3. A. A 10 μm thick cross-section of the ventral region of an unstained *Ophiopsila californica* arm. The lateral sides of the ventral plate (vp) and the lateral edges of the radial nerve cord (RNC) display fluorescence. The nonluminescent hyponeural nerve cord (hnc), vertebral muscles (vm), and radial water canal (rc) are also shown. The bar represents 100 μm. B. A 10 μm thick cross-section of *Ophiopsila californica*, unstained, at the level of the spines (s). Fluorescent varicosities (v) are seen in the spines and the processes (p) are also seen within the spinal nerves (sn). The bar represents 100 μm.

distribution of luminescent areas observed by visual means and image intensification. Furthermore, the fluorescence intensity of an area was directly related to the *in vivo* capability of that area to produce light. The long term stability of the

fluorescence in *O. californica* allowed a more precise identification of the luminescent areas than could be accomplished using the luminescence itself.

Figure 1 illustrates the location of the luminescent sites within several segments of an *O. californica* arm. All of the lateral spines along the arm are luminescent. However, the greatest intensity of luminescence, as judged by fluorescence, is in the basal and ventral region of each spine (Fig. 2a). Much of the lateral plate is luminescent, but the ventral portion of each plate is more luminescent than the dorsal portion. The proximal end of the lateral plate tends to be brightly luminescent, whereas the distal end is only weakly luminescent (Fig. 2a). In the distal region of the arm, the lateral plates tend to wrap around the nonluminescent dorsal plate and meet, thus resulting in a dorsal luminescence that is not present in the proximal arm. Also, all regions of the ventral plate are luminescent. Both pairs of tentacular scales are luminescent. The large scales exhibit a broad luminescence basally, which tapers to a narrow luminescent central core extending approximately half the length of the scale. The smaller tentacular scales are uniformly luminescent. Elements within the lateral region of the radial nerve cord are also luminescent; however, they are restricted to the ganglionic regions of the nerve cord (Fig. 3a). Both the lateral nerves and spinal nerves have luminescent tissue associated with them (Fig. 3b).

As noted in the preliminary paper (Brehm *et al.*, 1973), image intensification studies indicated that the intensity of luminescence in *O. californica* is related to the pigmentation pattern along the arm (Fig. 2b). Brown and tan bands alternate along the dorsal part of the arm, and each is separated by a narrow white band. The two darker bands include several arm segments, while the white bands generally include a single segment. The white band emits the most intense luminescence in the intact animal. This segment, when examined after KCl treatment, is the most intensely fluorescent region. The brown band has the weakest luminescence and fluorescence, while the intensity of the tan band is intermediate to the brown and white bands. Furthermore, it has been observed that the threshold of electrical excitability is related to the pigmentation of the segment. When the animal luminesces, tan bands can be observed to luminesce all along the arm length while brown bands often remain totally unlit (Fig. 2b).

Amphipholis squamata. This species differs from *Ophiopsila californica* in the distribution and stability of the fluorescence. Visual comparisons demonstrated that, as in *O. californica*, fluorescence is equivalent to luminescence. In *A. squamata* the luminescence and fluorescence is restricted primarily to the lateral plates (Fig. 4). Both the dorsal and ventral plates show a small amount of fluorescence, indicating that they are only weakly luminescent (Fig. 4). Similarly, the oral plates occasionally show a small amount of fluorescence. The spines are not luminescent or fluorescent. A high degree of variability exists between *A. squamata* individuals with respect to fluorescent distribution. In the New Hampshire individuals, luminescence was substantially weaker and fluorescence more sparsely distributed than in California individuals. In both populations sampled, and unlike *Ophiopsila*, the fluorescence was very labile under xenon, mercury, and even tungsten light. Blue light from a xenon source applied to the arms for three to five minutes generally produced an irreversible disappearance of all fluorescence.

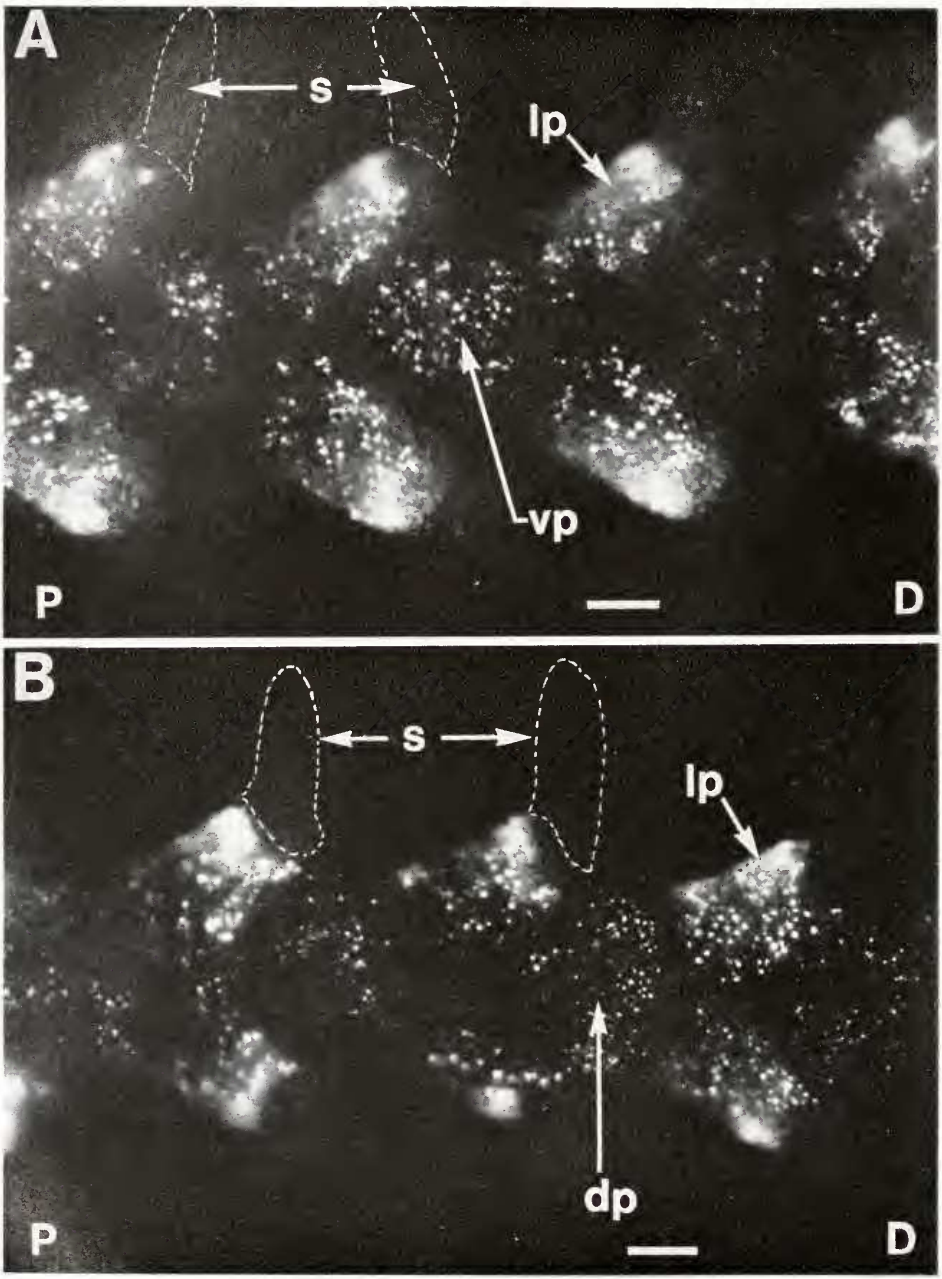

FIGURE 4. A. A ventral view of the fluorescence within four segments of an *Amphipholis squamata* arm. The arm was pretreated with 0.54 M KCl. The lateral plates (lp), ventral plate (vp), and spines (s) are labelled. The nonfluorescent spines are outlined in two segments. The proximal (P) and distal (D) segments are also indicated. B. A dorsal view of the same four segments of *Amphipholis squamata*. The lateral plates (lp), spines (s), and dorsal plate (dp) are labelled. The nonfluorescent spines are outlined in two segments. The proximal (P) and distal (D) segments are shown. The bar represents 100 μm in A and B.

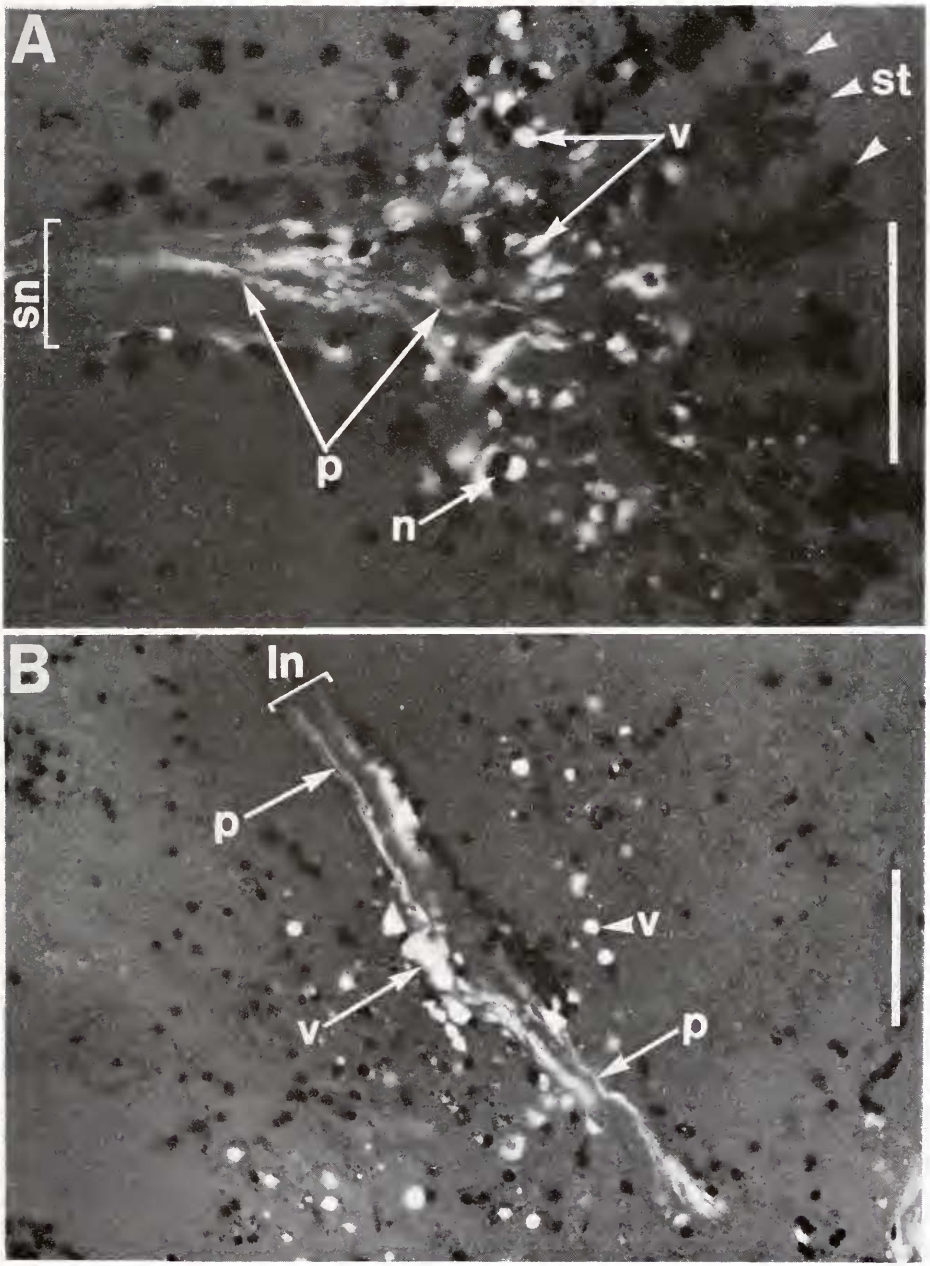

FIGURE 5. A. A longitudinal 10 μm thick section of *Ophiopsila californica* spine stained with Harris hematoxylin. Fluorescence is seen in the varicosities (v) as well as in the processes (p). The nuclei (n) of epithelial cells stain dark. The spinal nerve (sn) containing luminescent processes branches within the spine and stops short of the spine tip (st with arrows). The bar represents 100 μm. B. A longitudinal 10 μm thick section of *Ophiopsila californica* lateral nerve cord (ln) stained with Harris hematoxylin. Luminescent processes (p) are seen within the lateral nerve cord and varicosities (v) are within the surrounding tissue. The bar represents 100 μm.

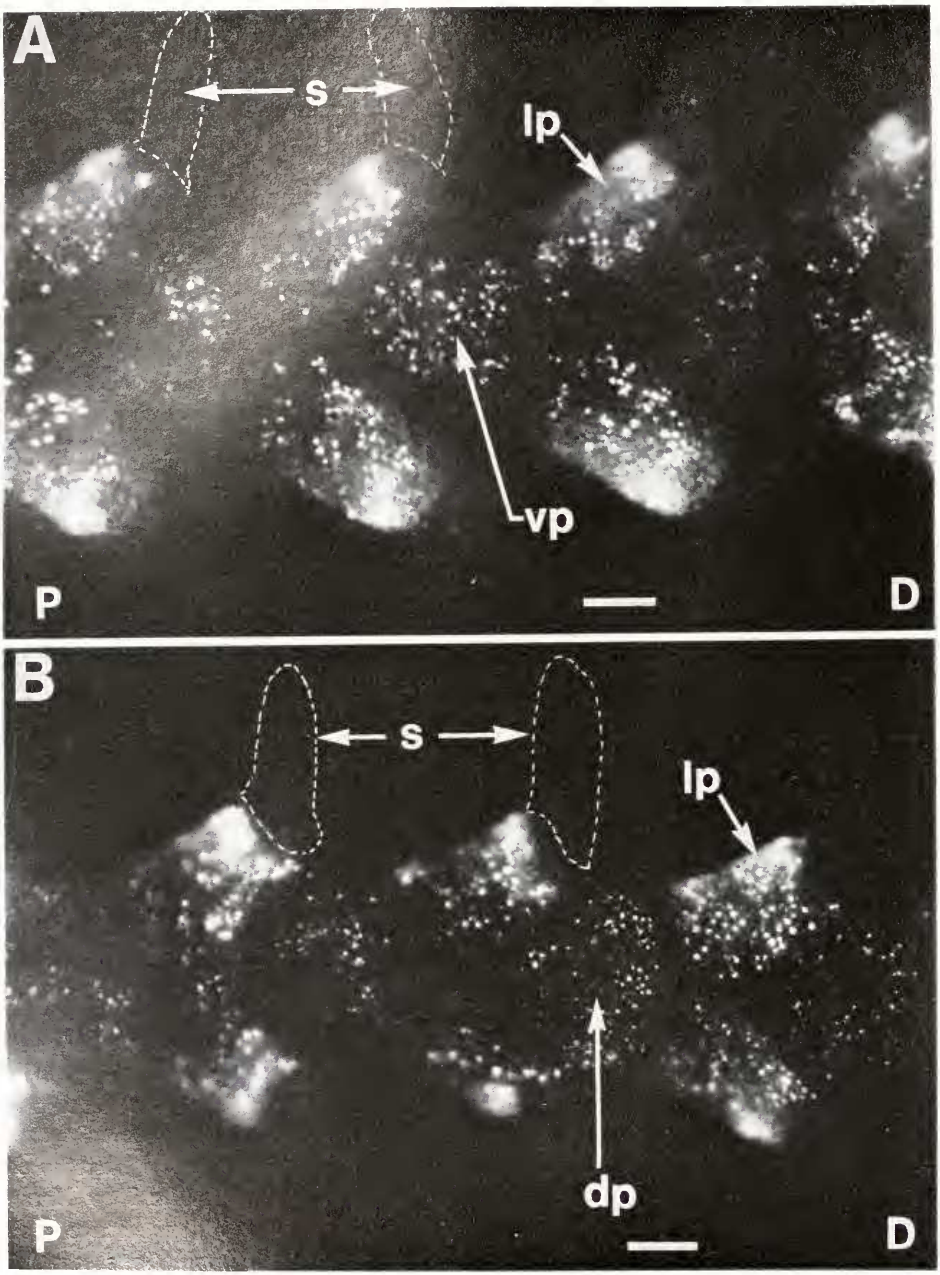

FIGURE 4. A. A ventral view of the fluorescence within four segments of an *Amphipholis squamata* arm. The arm was pretreated with 0.54 M KCl. The lateral plates (lp), ventral plate (vp), and spines (s) are labelled. The nonfluorescent spines are outlined in two segments. The proximal (P) and distal (D) segments are also indicated. B. A dorsal view of the same four segments of *Amphipholis squamata*. The lateral plates (lp), spines (s), and dorsal plate (dp) are labelled. The nonfluorescent spines are outlined in two segments. The proximal (P) and distal (D) segments are shown. The bar represents 100 μm in A and B.

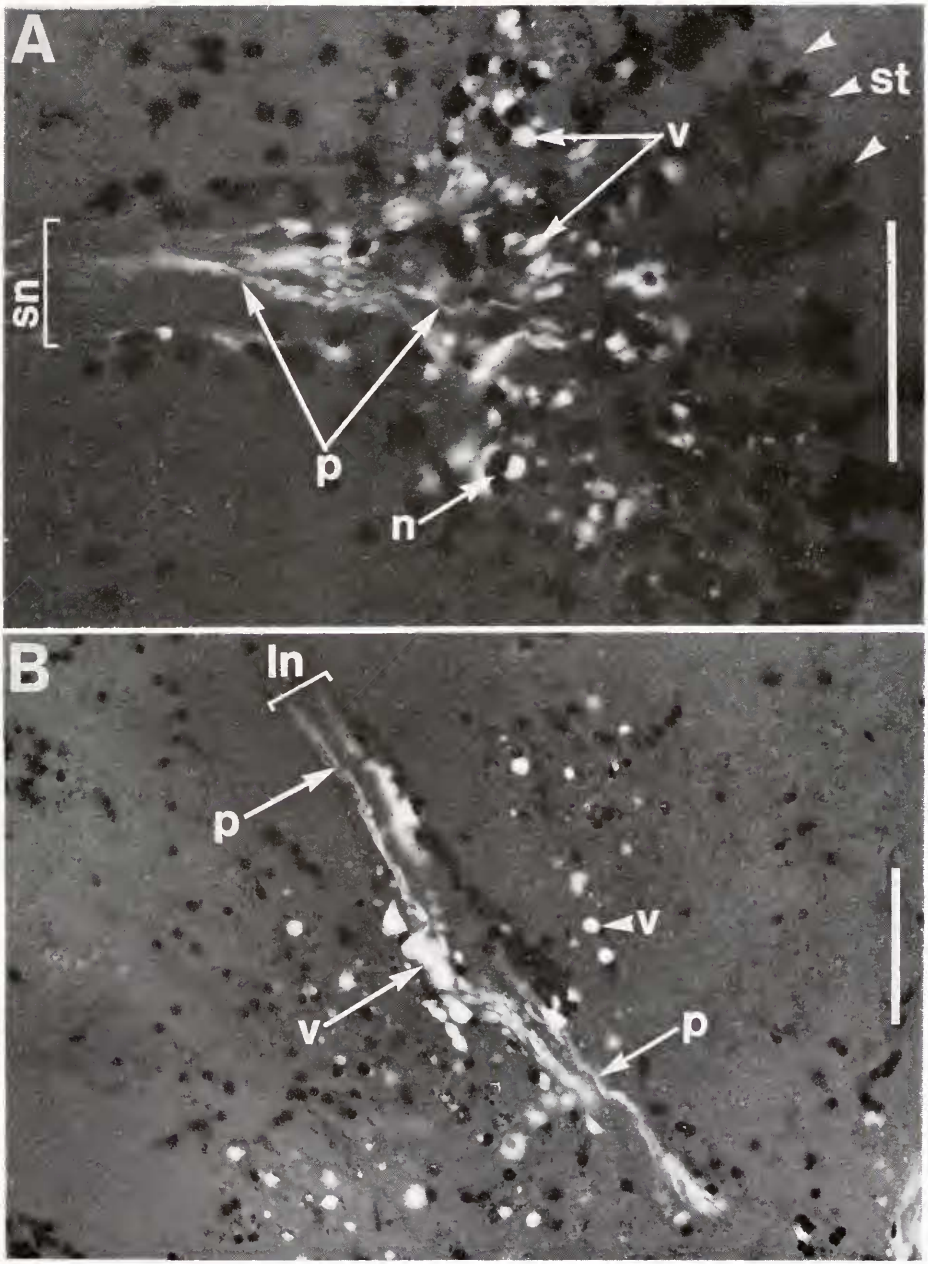

FIGURE 5. A. A longitudinal 10 μm thick section of *Ophiopsila californica* spine stained with Harris hematoxylin. Fluorescence is seen in the varicosities (v) as well as in the processes (p). The nuclei (n) of epithelial cells stain dark. The spinal nerve (sn) containing luminescent processes branches within the spine and stops short of the spine tip (st with arrows). The bar represents 100 μm. B. A longitudinal 10 μm thick section of *Ophiopsila californica* lateral nerve cord (ln) stained with Harris hematoxylin. Luminescent processes (p) are seen within the lateral nerve cord and varicosities (v) are within the surrounding tissue. The bar represents 100 μm.

Morphology of the photocytes

Fluorescence, associated with luminescent tissue, was maintained through fixation, decalcification, dehydration, and staining and could be detected in cross sections of *O. californica* (Figs. 3 and 5). Decalcification eliminated the light scattering effect of the skeleton and allowed a precise identification of the photocytes. The photocytes appear to be composed of long narrow processes which have occasional swellings, termed varicosities. Thus, the photocytes have two morphological components: varicosities and associated processes (Fig. 5). Both tend to be deep in the skeleton, but are also found in association with the nervous system. The relationship between varicosities and processes is not clear. Moreover, the boundaries of a single photocyte cannot be determined, so it is not known how many varicosities and processes constitute a single cell.

The varicosities are usually spherical and are frequently drawn out to meet an associated process, the second morphological component (Fig. 5). The varicosities average 3.0 μm in diameter with a range of 0.4 to 16 μm. Below 0.4 μm the varicosities become indistinguishable from the processes. There are thousands of these varicosities within one segment of the arm. A small percentage of the varicosities are nucleated, as judged from hematoxylin staining, and tend to be the largest within the size range. The varicosities lie amongst the cellular matrix which ramifies through the skeletal material (Fig. 5). The highest density of the varicosities is in the region immediately surrounding the ganglion at the base of each spine. The largest varicosities are found in this same area.

The processes have a diameter of less than 0.4 μm and appear to have their origin in the lateral edges of the radial nerve cord (Fig. 3a). It has been observed through electron microscopy (personal observation) that the hyponeural nerve cord wraps around the lateral edge of the radial nerve cord. Therefore, some of the observed fluorescence may also be associated with this region of hyponeural tissue. The processes are restricted to the region of radial nerve cord which is proximal to the origin of the lateral nerve cord (Fig. 1). In each segment at the end of the radial nerve cord ganglia, the processes appear to change direction and run within the lateral nerve cord (Figs. 3a and 5b). Except for their bright fluorescence, the processes are indistinguishable from the accompanying nervous tissue at the light microscopic level. The processes extend from the lateral nerve cord into the nerve bundle of the spines and can be followed as they branch off to adjoin varicosities within the spines (Figs. 3b and 5a). Processes are not always associated with nerve bundles. They are seen to connect adjacent varicosities in every luminescent area.

Varicosities and their processes, henceforth referred to as photocytes, are also present in the ventral plate (Fig. 3a), lateral plate (Fig. 5b), and tentacular scales (Fig. 2a). The absence of photocytes in nonluminescent regions such as the vertebral ossicle, dorsal plate, musculature, and the tube feet is conspicuous. The photocytes do not stain with Harris hematoxylin, toluidine blue, or thionin and are therefore quite transparent to transmitted tungsten light. They do, however, show a slight affinity for Holme's silver stain. The photocytes have never been observed to reach the surface cuticle.

Examination of the fresh tissue of *Amphipholis squamata* indicates that a similar network of varicosities and processes is responsible for the luminescence in

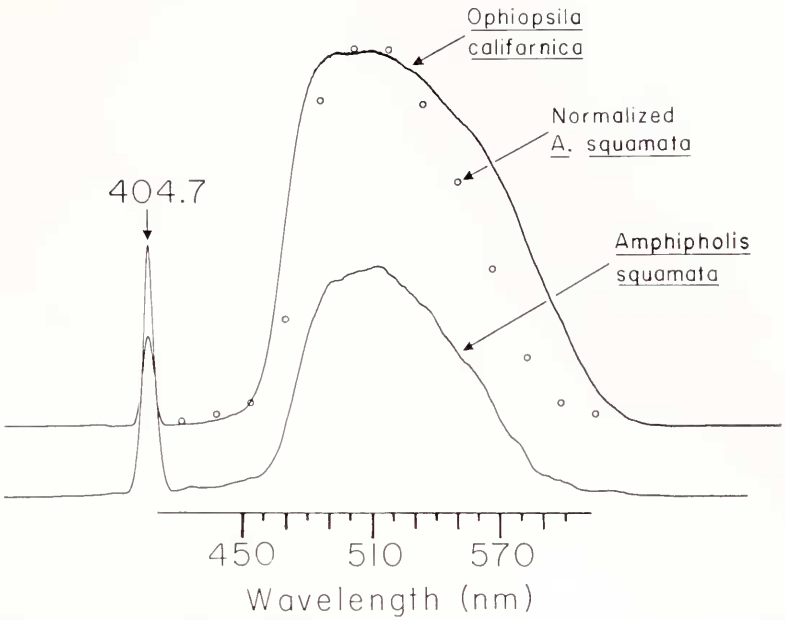

FIGURE 6. The luminescent emission spectra of *Ophiopsila californica*, *Amphipholis squamata*, and *Amphipholis squamata* normalized to the same peak amplitude as *Ophiopsila californica* (open circles). The ordinate indicates relative intensity in arbitrary units and the 404.7 nm peak is a calibration mark.

this species (Fig. 4). Since the fluorescence is less stable in this species than *O. californica*, similar histological studies were not attempted.

Emission spectra

The luminescent emission spectra of *Ophiopsila californica* and *Amphipholis squamata* (Fig. 6) are extremely broad with a similar approximate maximum at 510 nm and a half band-width of 71 nm. The emission spectra did not differ with chemical, mechanical, or electrical stimulation. In *O. californica* there was also no detectable difference in emission spectra from tan or brown pigmented bands. The spectra were not fully corrected for system nonlinearities, but the peak and qualitative features such as breadth are reliable.

The green emission of the *in vivo* fluorescence is visually identical to that of the luminescence.

DISCUSSION

The luminescent emission spectrum reported for *O. californica* in this paper has a peak at 510 nm and lacks pronounced shoulders. This differs from the previous report of a peak at 525 nm with a pronounced shoulder at 485 nm (Brehm *et al.*, 1973). These differences in spectra can only be ascribed to variability between animals. Wampler (1977), using as yet unidentified species of ophiuroids, reported a peak at 518 nm with shoulders at approximately 496 nm

TABLE I

Historical findings on luminescent ophuroids.

Species	Sites of luminescence	Color of emission	Source
Acrocnida branchiata	lateral plates	bluish-white	Gotto, 1963
Amphipholis (=*Amphiura*) *squamata*	vertebral muscle	yellow-green	Quatrefages, 1843
	base of tube feet	green	Panceri, 1878
	tube feet	green	Sterzinger, 1907
	lateral plates	yellow-green	Mangold, 1908
	lateral plates	yellow-green	Reichensperger, 1908a, b
	base of spines and lateral plates	—	Trojan, 1909a
	base of spines	—	Buchanan, 1963
Amphiura filiformis	entire spines	green	Mangold, 1907
	entire spines	green	Reichensperger, 1908a, b
	entire spines	—	Buchanan, 1963
Amphiura kandai	—	blue-green	Kato, 1947
Ophiocantha aculeata	lateral plates	blue-green	Herring, 1974
Ophiocantha bidentata (=*spinulosa*)	tube feet and ventral plates	—	Trojan, 1908, 1909b
	spines and lateral plates	yellow-green	Sokolow, 1909
	lateral plates	blue	Herring, 1974
Ophiopsila annulosa	ventral plate, proximal lateral plates, spines, tentacular scales	yellow-green	Mangold, 1907
	ventral plate, spines, proximal lateral plates, tentacular scales	yellow-green	Reichensperger, 1908a, b
	lateral plate, ventral plate, tentacular scales	green	Trojan, 1908, 1909b
Ophiopsila aranea	ventral plate, proximal lateral plates	green	Mangold, 1907
	—	bluish-green	Trojan, 1908, 1909b
	ventral plate, proximal lateral plates	green	Reichensperger, 1908a, b
Ophiopsila californica	ventral plate, lateral plates, spines, tentacular scales, radial shields	green	Brehm *et al.*, 1973
Ophioscolex glacialis	spines		Sokolow, 1909

and 552 nm. Interspecific variation in emission spectra is also suggested by the blue luminescence of *Ophiocantha bidentata* (Herring, 1974). Table I also indicates that blue luminescence may be seen in some other ophiuroids as contrasted with the visually green luminescence of *O. californica* and *A. squamata*. A blue luminescence was also observed in holothuroids and asteroids (Herring, 1974) and certain holothuroids show a peak emission at 472 nm (Wampler, 1977). It would be interesting to investigate the biochemical basis of visually observable differences in emission spectra which exist within the Ophiuroidea and other echinoderms.

In *O. californica* a green fluorescence was found, which is visually identical in color to the luminescent emission. The fact that fluorescence occurs after luminescence suggests that an oxidation product of the reaction is responsible for the fluorescence.

Historically, the statements concerning the distribution and identification of the photocytes in ophiuroids have been contradictory. Table I indicates some of the confusion surrounding the question of which areas within a given ophiuroid are luminescent. In this paper the question over both the cellular origin of luminescence and distribution of photocytes has been resolved in *O. californica* and *A. squamata*. However, the findings for all other species shown in Table I

should remain suspect until techniques such as image intensification and fluorescence microscopy have been applied to them.

Fluorescence microscopy has proven to be the most valuable technique for examination of the photocytes in *O. californica* and *A. squamata.* Harvey (1952) reported that in ultraviolet light *A. squamata* and *O. aranea* show a yellowish green fluorescence, but he did not give a description of its distribution, except to note (p. 479) that it was "evenly distributed over the plates and . . . on the feet" in *O. aranea.* He also noted (p. 479) that in *A. squamata* the fluorescence appears as a "network of yellowish green fluorescent material on the plates". Using ultraviolet radiation, we have been able to detect only a weak luminescence-associated fluorescence in both *O. californica* and *A. squamata.* However, such fluorescence can be easily observed in both of these species by exciting with a longer wavelength blue light. This method has also proven successful on many luminescent coelenterates (Morin and Reynolds, 1974). In this paper fluorescence was utilized in conjunction with standard histological techniques to examine individual photocytes. Thus the technique of fluorescence microscopy has allowed identification of both the spatial distribution and morphology of photocytes in these two species of ophiuroids.

The photocytes described here for *O. californica* bear no resemblance to those described by other authors. The varicosities in *O. californica* are much smaller and are more numerous than the photocytes described in any other ophiuroid species. The photocyte processes do not extend to the surface of the spines. Furthermore, the photocytes do not stain with thionin (Reichensperger, 1908b; Buchanan, 1963) or toluidine blue (Buchanan, 1963), as previously reported. We found large gland cells in the spines, which correspond to the cells described by earlier workers. These gland cells neither match the distribution of photogenic tissue nor are they fluorescent. It can be concluded, therefore, that this discussion concerns a newly described cellular origin of luminescence in the genus *Ophiopsila.* The luminescence emanates from varicosities and associated processes and not from a glandular cell type in *O. californica.* A similar morphology is suggested in *A. squamata.* Herring (1974) has found photocytes in the holothuroids and asteroids which may bear similarities to those described here for *O. californica.*

The fact that at the light microscopical level the processes are morphologically indistinguishable from the surrounding nervous tissue is intriguing. Physiologically, it can be demonstrated that propagation of luminescence along the arm is under the direct control of the radial nerve cord (personal observations). These observations suggest the following possibilities. First, the photocyte processes may represent greatly elongated extensions of effector cells which reach to the radial nerve cord. The phenomenon of effector processes approaching axons as a method of innervation in echinoderms has been reported several times. In the tube foot-ampullary system of the asteroid *Astropecten irregularis,* long muscle processes from the ampullary seam extend to internuncial neurons in the radial nerve cord (Cobb, 1967). Also, in the tridentate pedicellaria of the echinoid *Echinus* (Cobb, 1968) and in the vertebral muscles of *Ophiothrix fragilis* (Pentreath and Cottrell, 1971), it has been shown that processes of the muscles pass to the axon bundles for innervation. Secondly, the luminescent processes may be of neural origin. The similar affinity for Holme's silver stain seen in